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p38 and p38 reprogram liver metabolism by modulating neutrophil infiltration

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   19 May 2015

Thank you for the submission of your manuscript entitled "p38γ and p38δ reprogram liver metabolism by modulating neutrophil infiltration". We have now received the reports from the referees, which I copy below.

As you can see from their comments, all three referees are to a certain extent supportive of the publication of your study in The EMBO Journal, but point out to a number of significant concerns, that will require considerable additional experimental work before we can accept your manuscript. I will not repeat here the referee concerns, which we believe, are clear, as they are their suggestions for improvement of the manuscript. In any case, please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems.

Given the extensive revision that need to be implemented, publication of the study in The EMBO journal cannot be considered at this stage. However, as all three referees state the potential interest of your findings, we would like to give you the opportunity to address the reviewers concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed in their reports) taken on board.
Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The role of p38γ/δ in non-alocoholic fatty liver disease (NAFLD) was investigated, since p38δ expression was increased in patient NAFLD livers. Mice lacking p38γ/δ expression were shown to be resistant to diet-induced liver steatosis and glucose intolerance. It is proposed that p38γ/δ expression in neutrophils is required for migration to the damaged liver.

NAFLD is one of the most common forms of liver disease in Western industrialized nations and is strongly associated with both insulin resistance and metabolic syndrome. Increased understanding of the signaling pathways involved is important as this may provide new approaches for therapeutic intervention. The study by Gonzalez-Teran et al. indicates a critical role for the atypical p38 isoforms in NAFLD. However, the evidence linking this directly to p38γ/δ regulation of neutrophil migration is somewhat unconvincing, and the underlying molecular mechanism by which p38γ/δ regulates neutrophil trafficking is not established.

Specific points

1. Analysis of liver biopsies (n = 5) from obese subjects indicates elevated p38δ protein expression (Figure 1A). More control/obese subjects should be analyzed, expression data quantified and differences tested for statistical significance. Does obesity increase p38α/β and p38γ protein expression in the liver? Does obesity increase liver p38γ and p38δ mRNA expression?

2. The effects of MCD diet on p38γ/δ protein expression in the liver should be quantified and differences tested for statistical significance (Figure 1B). In addition, the effect of MCD diet on p38α/β proteins should be determined. As for point 1, liver mRNA expression of p38 isoforms should also be assayed.

3. In Figure 1E, MCD diet induces a significant increase in ALT in WT mice (ND/MCD comparison). Is this difference significant in p38γ/δ-/- mice? This is also relevant for subsequent datasets in which only the statistical significance of difference between WT and p38γ/δ-/- mice is only shown for the MCD samples.

4. The text should be altered to make it clear that the effect of p38γ/δ-deficiency on the recruitment of myeloid cells to the liver was determined by flow cytometry in Figure S2. Similarly for the text describing Figure 2E.

5. What is the efficiency of deletion of p38γ/δ in myeloid cell populations (macrophages, neutrophils and DCs) in the liver and spleens of p38γ/δLyzs-KO mice?

6. Figure 4C is incorrectly cited in the text, which refers to lower circulating ALT levels.

7. Figure S5 shows increased neutrophil activation in liver biopsies from a single obese patient compared to control, as judged by nitrosamine staining. This result would appear to be meaningless. Multiple patient/control biopsies need to be analyzed, nitrosamine staining quantified and differences tested statistically.

8. Figure 6 D and E show that p38γ/δ-/- neutrophil numbers in the steatotic liver were reduced compared to WT after injection of DiO/DiD-labeled cells. The authors claim that this difference is due to reduced migration by the p38γ/δ-/- cells. Can it be ruled out that p38γ/δ deficiency impairs neutrophil survival? Why are some cells apparently labeled with both DiO and DiD?

9. Figure 6F indicates that p38γ/δ-deficient neutrophils displayed increased rolling and decreased adhesion, which correlated with higher L-selectin and lower CD11b expression. This section would be substantially improved if the functional importance of p38γ/δ-dependent alterations in L-selectin and CD11b expression levels for neutrophil rolling/adhesion were tested directly (by knockdown / over-expression).

10. Anti-Ly6G treatment significantly reduced triglycerides and ALT concentrations in both control Lysz-Cre and p38γ/δLyssz-KO mice (Figure 7 C and D). This would appear to indicate that neutrophils were required for MCD-induced liver steatosis and necrosis whether or not p38γ/δ was expressed in myeloid cells.

11. The Cxcl1 data shown in Figure 7E are not mentioned in the text. Does anti-Ly6G treatment affect Il6 expression levels?

12. RNAseq revealed substantial overlap of hepaptic gene expression in anti-Ly6G treated control and p38γ/δLyss-KO mice (Figure 7D). Gene ontology analyses revealed that the overlapping genes were linked to elevated oxidative lipid metabolism and decreased inflammation. This correlation is consistent the authors' hypothesis that p38γ/δ deficiency reduces NAFLD by impairing neutrophil recruitment to the steatotic liver. However, the study falls short of demonstrating how p38γ/δ signaling regulates neutrophil trafficking.

Referee #2:

The authors demonstrate an important role for p38d/g for NAFLD/NASH. They propose that p38 regulates hepatic neutrophil infiltration and mediates hepatic steatosis/steatohepatitis, insulin resistance, but not obesity/body weight gain after HFD/MCD diet.

1. F4/80 as marker for hepatic macrophages is lower in livers after MCD in p38d/g-Lyzs-Ko mice. Some of these macrophages are resident (Kupffer cells) and don't need to migrate like neutrophils. A careful characterization of the p38d/g-Lyzs-Ko mouse is required. Do isolated hepatic macrophages/Kupffer cells express p38d/g? If Kupffer cells isolated from p38d/g-Lyzs-Ko do not express p38d/g, I'm not convinced that the effect seen is mediated by neutrophils. Kupffer cells might secrete a neutrophil attractant that is absent in p38d/g-Lyzs-Ko mice, which is acknowledged in the Discussion. Or they might just secrete less cytokines and chemokines, which makes the mouse resistant to steatosis. This needs to be carefully addressed, beyond a competitive migration assay.

2. The MCD model is a problematic model for NASH as mice do poorly and lose weight as opposed to humans with NASH. A CDAA or fast-food diet model (PMID: 21836057) is closer to human NASH. One of these models should be added. In particular, because hepatic TGs in p38d/g-Lyzs-Ko mice on HFD are lower, but not significantly lower (Fig. 4A), which is problematic since the entire paper is based on liver steatosis. Is this a type 2 error, meaning is this experiment underpowered? This could be easily confirmed with a fast-food diet, which develops robust steatosis rather than minimal steatosis following HFD for 10 weeks.

3. Hallmarks of NASH include hepatocyte ballooning and fibrosis. Please have a pathologist evaluate for hepatocyte ballooning and evaluate fibrosis on the gene and protein level (Sirius red staining, collagen, SMA and Timp-1 qPCR).

4. Absolute values for hepatic TGs are dramatically different between experiments. Baseline levels are between 1 to 25mg/g tissue. Something is wrong with this assay. Please repeat and confirm all measurements and provide evidence that results are reproducible.

5. Fig2E: I assume hepatic tissue was evaluated, please state either in the results or figure legends.

6. Fig. 3A: please add BW for mice on ND.

Referee #3:

Using myeloid cell-specific inactivation of the p38γ & δ isoforms and neutrophil depletion by antibody administration in a methionine choline deficient (MCD) and HFD-models of steatosis, Gonzalez Terran report that neutrophil expression of these isoforms is involved in the development of non alcoholic fatty liver disease (NAFLD) and that neutrophil infiltration into the liver

contributes to steatosis. Such findings are corroborated by increased p38δ expression in liver from NAFLD patients.

Major comments

-As at least 2 articles (see below) have already reported a role for neutrophils in NASH, the involvement of neutrophils in NAFLD, a related liver disease is not entirely new.
Rensen, S.S et al.. Increased hepatic myeloperoxidase activity in obese subjects with nonalcoholic steatohepatitis. Am. J. Pathol. 2009, 175, 1473-1482.
Rensen, S.S et al. Neutrophil-derived myeloperoxidase aggravates non-alcoholic steatohepatitis in low-density lipoprotein receptor-deficient mice. PLoS One 2012, 7, e52411.

-The characterization of NAFLD in the patient study is lacking. The authors provide no information on the patients, the histological scoring of their livers and diagnosis of NASH (including ballooning and inflammation). Moreover, there are no data on p38g in human livers nor information whether the changes in p38 isoforms are associated with markers of p38 activation and the different components of histological NASH scoring. As such the human data are very incomplete.

- While the metabolic phenotype of of p38γ/δ LysM/LysM mice is extensively described, the immunological part is overlooking some important points.
a. LysozymeM promoter-driven expression of Cre recombinase achieves myeloîd inactivation of floxed genes. Due to the essential contribution of macrophages, Kupffer cells, etc.to steatosis, the extent of p38γ and δ deletion in all these cells types, as well as in neutrophils should be addressed. Use of other myeloid cells not normally expressing LysM such as dendritic cells as control is also advisable. Additionally, what is the status of LysM expression in monocytes?
b. Figures 6A and S1. The mere identification of liver myeloïd cells on the basis of CD11b and Gr1 is insufficient. Figure 6B-C. More specific markers should be used and potential alterations of macrophage polarization status should be documented, at least using surface markers.
c. Figure 6D-F. Similar experiments should be performed with monocytes.
d. Figure 7. Saline is not the proper control for Ly6G administration. An isotype control antibody should be used. Administration protocol of antibody is not described in the methods section. How was the depletion efficiency assessed?
e. Figure S5. Title is misleading as the presented data in humans do not show that "Neutrophils control liver metabolic changes in steatosis development". Overall, the limited data presented about neutrophils in humans do not really support the the authors' claims about the relevance of findings to humans, which rather stems from previously published work. A reference describing that anti-nitroseamine antibody specifically identifies neutrophils (and for example does not also label eosinophils which also have a very active oxidative metabolism) is missing.

-Figure 8 and the gene expression analysis strategy is difficult to follow. Figure 8E does not refer to a GO analysis as stated in the text, and Figure 8F and 8G are not described/explained. Conclusions drawn from Figure 8 are overstated as gene expression only reflects a potential implication of lipid oxidation and other processes to the observed phenotype.

- How were data from the different platforms (GAII and HiSeq) normalized with respect to the number of reads? What is the point of running paired-end sequencing on a 36 bp-long sequence? How were Gene Ontology classifications performed? A detailed explanation of data processing is mandatory.

- Discussion is rather a summary of the results and should be extensively shortened and modified to properly put the results in the context of previously published work and of new perspectives.

-Figures 3A, 4C,E: show the chow diet controls also.
-Figure 4: panel labelling is off.
-Figure 4E: there appears no difference in insulin sensitivity (similar dynamic curves), and the difference is purely related to fasting glucose differences.

-Figure 8A-C: if lipid oxidation changes, why is there no difference in RER between WT and KO mice (Figure 3E)? Altogether, the mechanism via which neutrophils regulate steatosis is not addressed at all.

1st Revision - authors' response   04 November 2016

We thank the editors and reviewers for their consideration of our manuscript "p38γ and p38δ reprogram liver metabolism by modulating neutrophil infiltration", and for their positive comments and detailed critique regarding our study. We are glad that they think that "The authors demonstrate an important role for p38γ/δ for NAFLD/NASH." and we think that we have fully addressed all the concerns that the editors and reviewers had with our manuscript. Therefore, we believe that the revised manuscript has been greatly improved as a result of this revision and we hope that it can be considered a candidate for publication in your journal.

Reviewers' comments:

Referee #1:

We agree with the referee 1 in that "increased understanding of the signalling pathways involved is important as this may provide new approaches for therapeutic intervention". In this revised version we have tried to provide further evidence to link neutrophil migration with the process reported in our study. We hope that thanks to his/her suggestion he/she will find the revised manuscript more convincing.

Specific points

1. Analysis of liver biopsies (n = 5) from obese subjects indicates elevated p38δ protein expression (Figure 1A). More control/obese subjects should be analyzed, expression data quantified and differences tested for statistical significance. Does obesity increase p38α/β and p38γ protein expression in the liver? Does obesity increase liver p38γ and p38δ mRNA expression?

We thank the review for this suggestion. We have recruited more patients and controls and we now present data from more than 70 obese patients who underwent bariatric surgery (body mass index > 35 kg/m², and 20 controls with BMI < 35 kg/m² who underwent laparoscopic cholecystectomy. We also stratified controls into two groups according to the presence of NAFLD. Therefore, we now include three groups of patients: obese NAFLD patients with BMI> 35 kg/m², controls with BMI < 35 kg/m² and NAFLD and controls with BMI < 35 kg/m² and no liver disease. RT PCR to study the expression of p38γ and p38δ detected overexpression of these kinases in obese patients with NAFLD compared with controls without NAFLD (Figure 1A) and also in controls with NAFLD versus controls without NAFLD (Figura 1B). We also analysed p38α/β expression, but this analysis falls outside the scope of this paper and we plan to present these data in a separate study. We also include more samples in the protein study that confirmed increased p38δ protein expression in obese patients with NAFLD. However, due to limited sample quantity and the lack of a good antibody we were unable to assay p38γ properly: although we detected p38γ in some patients, we did not detect it in samples from many control patients and because of that these data were not available for analysis. Considering all our new data, we believe that these human results support an overexpression of p38γ and p38δ in patients with NAFLD and a potential role of these kinases in this disease.

2. The effects of MCD diet on p38γ/δ protein expression in the liver should be quantified and differences tested for statistical significance (Figure 1B). In addition, the effect of MCD diet on p38α/β proteins should be determined. As for point 1, liver mRNA expression of p38 isoforms should also be assayed.

We have now tested the expression and phosphorylation of p38γ and p38δ and quantified the blots. We found elevated p38γ and p38δ activation after MCD diet and a tendency of a increase of the protein levels of p38δ . The results are shown in Figure 1F. We also assay liver mRNA expression of p38γ and p38δ isoforms and found that mRNA form p38δ is elevated after MCD diet (Figure 1 D).

3. In Figure 1E, MCD diet induces a significant increase in ALT in WT mice (ND/MCD comparison). Is this difference significant in p38γ/δ-/- mice? This is also relevant for subsequent datasets in which only the statistical significance of difference between WT and p38γ/δ-/- mice is only shown for the MCD samples.

We only included the statistical differences between WT and KO groups to avoid overcrowding the figures. However, as the referee suggested we have included the rest of the statistical differences in this version of the paper.

4. The text should be altered to make it clear that the effect of p38γ/δ-deficiency on the recruitment of myeloid cells to the liver was determined by flow cytometry in Figure S2. Similarly for the text describing Figure 2E.

We have corrected this point in the text.

5. What is the efficiency of deletion of p38γ/δ in myeloid cell populations (macrophages, neutrophils and DCs) in the liver and spleens of p38γ/δLyzs-KO mice?

Because it is difficult to detect these kinases by immunoblotting in mouse samples, we have assayed this deletion by PCR, finding efficient deletion of the kinases using the Lyzs Cre system in these cell types.

6. Figure 4C is incorrectly cited in the text, which refers to lower circulating ALT levels.

Thanks for the comment, we have corrected this issue.

7. Figure S5 shows increased neutrophil activation in liver biopsies from a single obese patient compared to control, as judged by nitrosamine staining. This result would appear to be meaningless. Multiple patient/control biopsies need to be analyzed, nitrosamine staining quantified and differences tested statistically.

We now include human neutrophil Defensins 1-3 as another neutrophil cell marker, since nitrotyrosine could also mark eosinophils. More importantly, we now assay 9 samples from obese patients with NAFLD and controls without NAFLD, all of them without apparent signs of inflammation by H&E staining. Even in the obese patients with NAFLD but no evidence of infiltration, immunostaining showed increased levels of neutrophils. We quantified these results, which are now shown in Figure S6.

8. Figure 6 D and E show that p38γ/δ-/- neutrophil numbers in the steatotic liver were reduced compared to WT after injection of DiO/DiD-labeled cells. The authors claim that this difference is due to reduced migration by the p38γ/δ-/- cells. Can it be ruled out that p38γ/δ deficiency impairs neutrophil survival? Why are some cells apparently labeled with both DiO and DiD?

We tested neutrophil survival by conventional DAPI staining followed by FACS analysis. We found no differences between genotypes (fig S8A). We have also repeated the experiment with lower amounts of DiO and DiD to avoid double positive cells. The results are shown in figure 7D.

9. Figure 6F indicates that p38γ/δ-deficient neutrophils displayed increased rolling and decreased adhesion, which correlated with higher L-selectin and lower CD11b expression. This section would be substantially improved if the functional importance of p38γ/δ-dependent alterations in L-selectin and CD11b expression levels for neutrophil rolling/adhesion were tested directly (by knockdown / over-expression).

Since neutrophils are short-lived cells and cannot be easily manipulated ex vivo, we have been unable to genetically alter these cells. We further lack the genetic tools in mice to specifically alter L-selectin or CD11b/Mac-1. For this reason we performed functional assays using recombinant E-selectin and ICAM-1, the ligands for L-selectin and CD11b; these experiments showed that the defect is due to these molecules (Fig S 8C)

Moreover, we now generated, p38d$^{Mrp8-KO}$ mouse model, in which p38δ is deleted specifically in neutrophils. Lack of p38δ in neutrophils is enough to reduce MCD-induced steatosis. These new data indicate that p38γ/δ signalling regulates neutrophil trafficking.

10. Anti-Ly6G treatment significantly reduced triglycerides and ALT concentrations in both control Lyzs-Cre and p38γ/δ$^{Lyzs-KO}$ mice (Figure 7 C and D). This would appear to indicate that neutrophils were required for MCD-induced liver steatosis and necrosis whether or not p38γ/δ was expressed in myeloid cells.

We agree that neutrophil migration to the liver is essential for the development of steatosis. However, our data indicate that p38γ/δ$^{Lyzs-KO}$ mice show no further improvement in steatosis when neutrophils are depleted, suggesting that the protection p38γ/δ$^{Lyzs-KO}$ is due to the lack of neutrophil migration. If another cell type were involved we would expect a further improvement in the KO animals. We now include two new experiments to demonstrate the importance of neutrophil migration for the phenotype:

1) *Parabiosis assay, in which the circulation of a p38γ/δ$^{Lyzs-KO}$ or Lyzs-Cre mouse is shared with WT partner mouse. In these experiments, neutrophils from the WT partner migrate to the p38γ/δ$^{Lyzs-KO}$ liver in greater proportion than WT neutrophils migrate to Lyzs-Cre liver, suggesting deficient migration of the p38γ/δ$^{Lyzs-KO}$ neutrophils. Most importantly, this effect is enough to induce an increase of steatosis in p38γ/δ$^{Lyzs-KO}$ mice (Figure S9).*

2) *We have now also generated mice lacking p38δ specifically in neutrophils (Mrp8-cre driver), finding that and found that these mice are protected against steatosis (Figure S10).*

Unfortunately, with the tools available at the moment we cannot rule out a contribution to the phenotype from p38γ expressed in macrophages. However, the data provide strong evidence that p38 γ/δ in neutrophils is required for migration to the liver and induction of disease. This point is now discussed in the text.

11. The Cxcl1 data shown in Figure 7E are not mentioned in the text. Does anti-Ly6G treatment affect Il6 expression levels?

We now mention this result in the text, and we also included data for IL6.

12. RNAseq revealed substantial overlap of hepaptic gene expression in anti-Ly6G treated control and p38γ/δLyss-KO mice (Figure 7D). Gene ontology analyses revealed that the overlapping genes were linked to elevated oxidative lipid metabolism and decreased inflammation. This correlation is consistent the authors' hypothesis that p38γ/δ deficiency reduces NAFLD by impairing neutrophil recruitment to the steatotic liver. However, the study falls short of demonstrating how p38γ/δ signaling regulates neutrophil trafficking.

The parabiosis study provides further evidence that p38γ/δ signalling controls neutrophil trafficking. Joining the circulations of WT and p38γ/δ$^{Lyzs-KO}$ mice allowed us to measure competitive migration to the liver and whether an increased influx of neutrophils from the WT mice into the KO livers would be enough to induce steatosis. The data demonstrate that infiltration of WT neutrophils to p38γ/δ$^{Lyzs-KO}$ livers is enough to exacerbate the steatosis, providing definitive confirmation to our original conclusion.

Further support is provided by the p38d$^{Mrp8-KO}$ mouse model, in which p38δ is deleted specifically in neutrophils. Lack of p38δ in neutrophils is enough to reduce MCD-induced steatosis. These new data indicate that p38γ/δ signalling regulates neutrophil trafficking.
We agree that it would be interesting to study how neutrophils regulate liver metabolism, but we consider this question to be beyond the scope of this paper.

Referee #2:

The authors demonstrate an important role for p38d/g for NAFLD/NASH. They propose that p38 regulates hepatic neutrophil infiltration and mediates hepatic steatosis/steatohepatitis, insulin resistance, but not obesity/body weight gain after HFD/MCD diet.

1. F4/80 as marker for hepatic macrophages is lower in livers after MCD in p38d/g-Lyzs-Ko mice. Some of these macrophages are resident (Kupffer cells) and don't need to migrate like neutrophils. A careful characterization of the p38d/g-Lyzs-Ko mouse is required. Do isolated hepatic macrophages/Kupffer cells express p38d/g? If Kupffer cells isolated from p38d/g-Lyzs-Ko do not express p38d/g, I'm not convinced that the effect seen is mediated by neutrophils. Kupffer cells might secrete a neutrophil attractant that is absent in p38d/g-Lyzs-Ko mice, which is acknowledged in the Discussion. Or they might just secrete less cytokines and chemokines, which makes the mouse resistant to steatosis. This needs to be carefully addressed, beyond a competitive migration assay.

We thank the referee for this suggestion. We previously confirmed that Lyzs Cre mice delete p38γ/δ in Kupffer cells (Gonzalez-Teran 2013). We now mention this in the Discussion of the current manuscript. Importantly, however, we now demonstrate that neutrophil-intrinsic deletion of p38δ (Mrp8-cre deleter) confers hepatic protection (Fig.S10). Thus, even though our data indicate that p38γ/δ have a cell autonomous effect, we cannot exclude that these kinases also affect the phenotype of other cells and the secretion of chemokines or other attractants. But any effects such as these would be independent of the function of neutrophils.
Due the limited availability of tools for working with neutrophils, we performed a parabiosis study (see answers to Referee 1, above for details). In this study we found that neutrophils from the WT partner migrate better than neutrophils lacking p38γ/δ (Fig S9).

2. The MCD model is a problematic model for NASH as mice do poorly and lose weight as opposed to humans with NASH. A CDAA or fast-food diet model (PMID: 21836057) is closer to human NASH. One of these models should be added. In particular, because hepatic TGs in p38d/g-Lyzs-Ko mice on HFD are lower, but not significantly lower (Fig. 4A), which is problematic since the entire paper is based on liver steatosis. Is this a type 2 error, meaning is this experiment underpowered? This could be easily confirmed with a fast-food diet, which develops robust steatosis rather than minimal steatosis following HFD for 10 weeks.

We thank the referee for this suggestion. We found that a fast food diet (high fructose diet) is quite good at inducing steatosis, and the new data confirm and strengthen our results. We found that p38γ/δ^{Lyzs-KO} mice are protected against HFF-induced steatosis, presenting lower basal glucose levels and less liver infiltration evaluated by a pathologist. These data are presented in Fig S4.

3. Hallmarks of NASH include hepatocyte ballooning and fibrosis. Please have a pathologist evaluate for hepatocyte ballooning and evaluate fibrosis on the gene and protein level (Sirius red staining, collagen, SMA and Timp-1 qPCR).

Now we have performed picrosirius red and Masson's trichrome staining in liver. We also measured collagen, SMA, and Timp-1, finding that lack of p38γ/δ protects mice against MCD-induced fibrosis. These results are shown in Fig 2.

4. Absolute values for hepatic TGs are dramatically different between experiments. Baseline levels are between 1 to 25mg/g tissue. Something is wrong with this assay. Please repeat and confirm all measurements and provide evidence that results are reproducible.

We apologize; there was a problem with the conversion of values TG/mg of liver. We have repeated the uncertain measurements and corrected these data in the new version. The data confirm the initial results.

5. Fig2E: I assume hepatic tissue was evaluated, please state either in the results or figure legends.

We did evaluate hepatic tissue. We have now clarified this in the text and also in the figure legends.

6. Fig. 3A: please add BW for mice on ND.
We now include these data in Figure 3A. We found no differences in BW between genotypes fed a normal diet.

Referee #3:

Using myeloid cell-specific inactivation of the p38g & d isoforms and neutrophil depletion by antibody administration in a methionine choline deficient (MCD) and HFD-models of steatosis, Gonzalez Terran report that neutrophil expression of these isoforms is involved in the development of non alcoholic fatty liver disease (NAFLD) and that neutrophil infiltration into the liver contributes to steatosis. Such findings are corroborated by increased p38d expression in liver from NAFLD patients.

Major comments
-As at least 2 articles (see below) have already reported a role for neutrophils in NASH, the involvement of neutrophils in NAFLD, a related liver disease is not entirely new.
Rensen, S.S et al.. Increased hepatic myeloperoxidase activity in obese subjects with nonalcoholic steatohepatitis. Am. J. Pathol. 2009, 175, 1473-1482.
Rensen, S.S et al. Neutrophil-derived myeloperoxidase aggravates non-alcoholic steatohepatitis in low-density lipoprotein receptor-deficient mice. PLoS One 2012, 7, e52411.

We thank the reviewer for pointing out these papers to us, of which we were indeed aware. The key new finding that we describe is that modulation of two stress kinases, p38g and p38d, is enough to control neutrophil migration and to confer protection to the liver against this disease. We believe that this offers the possibility to use these kinases as a target for the treatment of steatosis.

-The characterization of NAFLD in the patient study is lacking. The authors provide no information on the patients, the histological scoring of their livers and diagnosis of NASH (including ballooning and inflammation). Moreover, there are no data on p38g in human livers nor information whether the changes in p38 isoforms are associated with markers of p38 activation and the different components of histological NASH scoring. As such the human data are very incomplete.

We had already included part of this information in Supplementary Table 1. We now include more information in this table. Moreover, we have substantially improved the human data. We now present data from more than 70 obese patients who underwent bariatric surgery (body mass index > 35 kg/m^2) and 20 controls with BMI < 35 kg/m^2 who underwent laparoscopic cholecystectomy. We also stratified controls into two groups according to the presence of NAFLD. Therefore, we now include three groups: obese NAFLD patients with BMI> 35 kg/m^2, controls with BMI < 35 kg/m^2 and NAFLD and controls with BMI < 35 kg/m^2 and no liver disease. Our results indicate that increased p38γ/δ expression is closely associated with steatosis. Even among patients with BMI < 35 kg/m^2, individuals with data of NAFLD also presented higher levels of p38γ when compared to controls without liver disease.

- While the metabolic phenotype of p38g/d LysM/LysM mice is extensively described, the immunological part is overlooking some important points.
   a. LysozymeM promoter-driven expression of Cre recombinase achieves myeloîd inactivation of floxed genes. Due to the essential contribution of macrophages, Kupffer cells, etc.to steatosis, the extent of p38g and d deletion in all these cells types, as well as in neutrophils should be addressed. Use of other myeloid cells not normally expressing LysM such as dendritic cells as control is also advisable. Additionally, what is the status of LysM expression in monocytes?

To examine the importance of p38g/d in neutrophils, we have performed 2 new studies:

1) We performed a parabiosis assay in which circulating blood of the KO is in contact with blood from a WT animal. We found that in this context the neutrophils of the WT arrive in a bigger

proportion to the p38γ/δ$^{Lyzs-KO}$ liver than to the Lyzs-Cre liver. Most importantly, this effect is enough to induce an increase of steatosis in p38γ/δ$^{Lyzs-KO}$ mice (Figure S9).

1. *We have generated mice lacking p38δ specifically in neutrophils (Mrp8-cre driver), finding that these mice are protected against steatosis (Figure S10).*

b. Figures 6A and S1. The mere identification of liver myeloïd cells on the basis of CD11b and Gr1 is insufficient. Figure 6B-C. More specific markers should be used and potential alterations of macrophage polarization status should be documented, at least using surface markers.

We thank the review for this comment. We checked that our analysis was accurate by sorting these cells and identifying cells as neutrophils, macrophages or monocytes: this is now included in Figure 7A.
Moreover, we have measured M1 and M2 macrophage markers, finding no significant differences. This is included in Figure S2 B

c. Figure 6D-F. Similar experiments should be performed with monocytes.

We have examined neutrophil and monocyte/macrophage migration in the parabiosis study. We found no differences in relative infiltration by mutant and WT macrophages; in contrast, neutrophils from the WT partner migrated better than neutrophils lacking p38γ/δ, indicating that the migratory defect is specific for neutrophils.

d. Figure 7. Saline is not the proper control for Ly6G administration. An isotype control antibody should be used. Administration protocol of antibody is not described in the methods section. How was the depletion efficiency assessed?

We have now included this in the methods section. To examine the depletion efficiency we took blood from these mice and checked the percentage of neutrophils in blood by flow cytometry.

e. Figure S5. Title is misleading as the presented data in humans do not show that "Neutrophils control liver metabolic changes in steatosis development". Overall, the limited data presented about neutrophils in humans do not really support the the authors' claims about the relevance of findings to humans, which rather stems from previously published work. A reference describing that anti-nitroseamine antibody specifically identifies neutrophils (and for example does not also label eosinophils which also have a very active oxidative metabolism) is missing.

Thank you for this comment. We changed the title of this figure, and include human neutrophil defensins 1-3 as another neutrophil cell marker, since nitrotyrosine could also mark eosinophils. More importantly, we now assay 9 samples from obese patients with NAFLD and controls without NAFLD, all of them without apparent signs of inflammation by H&E staining. Even in the obese patients with NAFLD but no evidence of infiltration, immunostaining showed increased levels of neutrophils. We quantified these results, which are now shown in Figure S7.

-Figure 8 and the gene expression analysis strategy is difficult to follow. Figure 8E does not refer to a GO analysis as stated in the text, and Figure 8F and 8G are not described/explained. Conclusions drawn from Figure 8 are overstated as gene expression only reflects a potential implication of lipid oxidation and other processes to the observed phenotype

We have now improved this section for clarity.

- How were data from the different platforms (GAII and HiSeq) normalized with respect to the number of reads? What is the point of running paired-end sequencing on a 36 bp-long sequence? How were Gene Ontology classifications performed? A detailed explanation of data processing is mandatory.

We have now improved this section for clarity.

- Discussion is rather a summary of the results and should be extensively shortened and modified to properly put the results in the context of previously published work and of new perspectives.
-

We have also rewritten parts of the Discussion to improve its content.

-Figures 3A, 4C,E: show the chow diet controls also.
Thanks, we now include these data.

-Figure 4: panel labelling is off.
Thanks, this has been corrected.

-Figure 4E: there appears no difference in insulin sensitivity (similar dynamic curves), and the difference is purely related to fasting glucose differences.

We agree that the main difference in this curve is the glucose level in the fed condition; for this reason we have removed this figure.

-Figure 8A-C: if lipid oxidation changes, why is there no difference in RER between WT and KO mice (Figure 3E)? Altogether, the mechanism via which neutrophils regulate steatosis is not addressed at all.

We were also surprised since we could not see any change in the RER of the metabolic cages. We think that the changes are not big enough for detection in a metabolic cage, because small changes in the metabolism of a specific organ could be missed by the total metabolism measured with this system.

2nd Editorial Decision   25 November 2015

Thank you for the submission of your revised manuscript to The EMBO Journal. As you will see below, your article was sent back to the referees, who now consider that you have properly dealt with the main concerns originally raised in the review process, and therefore I am writing with an 'accept in principle' decision. This means that I will be happy to formally accept your manuscript for publication once a few more minor issues have been addressed.

As I said, referees now believe that all major concerns have been addressed and your manuscript is almost ready for publication (see below). A few relatively minor points are still pending according to referee #1 and they will require mostly text modifications and clarifications.

In addition, browsing through the manuscript myself I have also noticed a few cosmetic issues that will need to be addressed in the final version of the paper. First, Supplementary information is now called "Appendix". This means that figure names must be changed to "Appendix S1", "Appendix S2", etc. Tables follow a similar pattern ("Appendix table 1", etc.). Please make sure that in-text call-outs to the figures are properly corrected. For maximum visibility, we do not publish supplementary references, and these should be included in the main text together with the main references.

I would also like to point out to a very minor issue with figure 1: the meaning of error bars in the figure is not explained in the legend as you properly do in all other figures. Thank you very much for your patience. I am looking forward to seeing the final version of your manuscript. Congratulations in advance for a successful publication.

REFEREE REPORT

Referee #1:

The authors have addressed most of my criticisms with the inclusion of new experiments in the revised manuscript. However, there are still a few issues remaining that need clarification.

Specific points

1. Analysis of liver biopsies (n = 5) from obese subjects indicates elevated p38δ protein expression (Figure 1A). More control/obese subjects should be analyzed, expression data quantified and differences tested for statistical significance. Does obesity increase p38α/β and p38γ protein expression in the liver? Does obesity increase liver p38γ and p38δ mRNA expression?

OK.

2. The effects of MCD diet on p38γ/δ protein expression in the liver should be quantified and differences tested for statistical significance (Figure 1B). In addition, the effect of MCD diet on p38α/β proteins should be determined. As for point 1, liver mRNA expression of p38 isoforms should also be assayed.

Figure 1E legend should read 'MCD or ND diet for the times indicated'. Vinculin is used as a loading control for p38γ and p38δ immunoprecipitates. How does that work?

3. In Figure 1E, MCD diet induces a significant increase in ALT in WT mice (ND/MCD comparison). Is this difference significant in p38γ/δ-/- mice? This is also relevant for subsequent datasets in which only the statistical significance of difference between WT and p38γ/δ-/- mice is only shown for the MCD samples.

OK.

4. The text should be altered to make it clear that the effect of p38γ/δ-deficiency on the recruitment of myeloid cells to the liver was determined by flow cytometry in Figure S2. Similarly for the text describing Figure 2E.

OK.

5. What is the efficiency of deletion of p38γ/δ in myeloid cell populations (macrophages, neutrophils and DCs) in the liver and spleens of p38γ/δLyzs-KO mice?

The authors have responded that the efficiency of deletion in myeloid cell populations was determined by qRT-PCR and that deletion was efficient. Where are these data? Are they referred to in the text?

6. Figure 4C is incorrectly cited in the text, which refers to lower circulating ALT levels.

OK.

7. Figure S5 shows increased neutrophil activation in liver biopsies from a single obese patient compared to control, as judged by nitrosamine staining. This result would appear to be meaningless. Multiple patient/control biopsies need to be analyzed, nitrosamine staining quantified and differences tested statistically.

In the revised manuscript, the authors have used Defensin 1-3 as an alternative marker for neutrophils and demonstrated a significantly increased fraction of positive cells in the livers of obese patients with NAFLD. Does Defensin 1-3 expression quantify neutrophil numbers or activation? Also, nitrosamine staining was not quantified as originally requested.

8. Figure 6 D and E show that p38γ/δ-/- neutrophil numbers in the steatotic liver were reduced compared to WT after injection of DiO/DiD-labeled cells. The authors claim that this difference is due to reduced migration by the p38γ/δ-/- cells. Can it be ruled out that p38γ/δ deficiency impairs neutrophil survival? Why are some cells apparently labeled with both DiO and DiD?

The authors state that there were no differences in neutrophil survival between different genotypes

based on the data shown in Supplementary Figure 7A. However, this would appear to show the cells that were injected at the beginning of the experiment. Also, the significance of the data in this figure are unclear since only DAPI negative cells (which by definition are alive) are shown. In addition, it is conventional in this type of experiment to perform a 'dye reversal' experiment, to confirm that the results are not being affected by specific toxic effects of Cy5-5 in p38γ/δ neutrophils. In my opinion, the revised paper still does not rule out some role for p38γ/δ in neutrophil survival and a statement to this effect should be added to the text.

9. Figure 6F indicates that p38γ/δ-deficient neutrophils displayed increased rolling and decreased adhesion, which correlated with higher L-selectin and lower CD11b expression. This section would be substantially improved if the functional importance of p38γ/δ-dependent alterations in L-selectin and CD11b expression levels for neutrophil rolling/adhesion were tested directly (by knockdown / over-expression).

The functional experiments investigating the effect of p38γ/δ deficiency on neutrophil binding to L-selectin and CD11b are a good addition to the study. The use of an Mrp8 Cre-driver to specifically delete p38γ/δ in neutrophils also potentially strengthens the conclusions of the study. However, this is dependent on the Mrp8 Cre-driver only deleting in neutrophils. It is important to confirm that p38γ/δ were not deleted in macrophages, which also have an important role in the steatosis model used. Assuming specific neutrophil deletion, the Mrp8-Cre data should be in the main manuscript and not in the Supplementary section.

10. Anti-Ly6G treatment significantly reduced triglycerides and ALT concentrations in both control Lysz-Cre and p38γ/δLyssz-KO mice (Figure 7 C and D). This would appear to indicate that neutrophils were required for MCD-induced liver steatosis and necrosis whether or not p38γ/δ was expressed in myeloid cells.

Point taken on the effect of neutrophil depletion with anti-Ly6G {plus minus} myeloid p38γ/δ expression.

11. The Cxcl1 data shown in Figure 7E are not mentioned in the text. Does anti-Ly6G treatment affect IL-6 expression levels?

OK.

12. RNAseq revealed substantial overlap of hepaptic gene expression in anti-Ly6G treated control and p38γ/δLyss-KO mice (Figure 7D). Gene ontology analyses revealed that the overlapping genes were linked to elevated oxidative lipid metabolism and decreased inflammation. This correlation is consistent the authors' hypothesis that p38γ/δ deficiency reduces NAFLD by impairing neutrophil recruitment to the steatotic liver. However, the study falls short of demonstrating how p38γ/δ signaling regulates neutrophil trafficking.

I am not arguing that the study does not establish that p38γ/δ regulates neutrophil trafficking. Rather, that the mechanism by which p38γ/δ mediate this function is not established. This is obviously an important area of research for the future.

Referee #2:

All my previous concerns have been adequately addressed.

Referee #3:

While the findings presented still do not bring a major advance over published work, the authors have improved significantly their manuscript. I leave it up to the editor to decide whether the priority of this manuscript for publication in the journal is sufficiently high.

Thank you for providing the positive reviews of our manuscript "p38γ and p38δ reprogram liver metabolism by modulating neutrophil infiltration".
We have meticulously revised the manuscript to address the questions raised by the reviewers, including all the points noted in your decision letter. We believe that the manuscript has been improved by the changes we have made, and we are very grateful to you and the reviewers for giving us this opportunity to revise our manuscript.

Reviewer 1's comments:

The authors have addressed most of my criticisms with the inclusion of new experiments in the revised manuscript. However, there are still a few issues remaining that need clarification.

Specific points

1. Analysis of liver biopsies (n = 5) from obese subjects indicates elevated p38δ protein expression (Figure 1A). More control/obese subjects should be analyzed, expression data quantified and differences tested for statistical significance. Does obesity increase p38α/β and p38γ protein expression in the liver? Does obesity increase liver p38γ and p38δ mRNA expression?

OK.

2. The effects of MCD diet on p38γ/δ protein expression in the liver should be quantified and differences tested for statistical significance (Figure 1B). In addition, the effect of MCD diet on p38α/β proteins should be determined. As for point 1, liver mRNA expression of p38 isoforms should also be assayed.

Figure 1E legend should read 'MCD or ND diet for the times indicated'. Vinculin is used as a loading control for p38γ and p38δ immunoprecipitates. How does that work?

The vinculin is detected by western blot using 30ul of the total lysates that are prepared for the IP, before the IP is performed, to be sure that we load the same amount of protein in all the immunoprecipitated samples. Now we specify this in the figure.

3. In Figure 1E, MCD diet induces a significant increase in ALT in WT mice (ND/MCD comparison). Is this difference significant in p38γ/δ-/- mice? This is also relevant for subsequent datasets in which only the statistical significance of difference between WT and p38γ/δ-/- mice is only shown for the MCD samples.

OK.

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OK.

5. What is the efficiency of deletion of p38γ/δ in myeloid cell populations (macrophages, neutrophils and DCs) in the liver and spleens of p38γ/δLyzs-KO mice?

The authors have responded that the efficiency of deletion in myeloid cell populations was determined by qRT-PCR and that deletion was efficient. Where are these data? Are they referred to in the text?

We are sorry, we missed to include it. Now we included these data in Appendix 3S.

6. Figure 4C is incorrectly cited in the text, which refers to lower circulating ALT levels.

OK.

7. Figure S5 shows increased neutrophil activation in liver biopsies from a single obese patient compared to control, as judged by nitrosamine staining. This result would appear to be meaningless. Multiple patient/control biopsies need to be analyzed, nitrosamine staining quantified and differences tested statistically.

In the revised manuscript, the authors have used Defensin 1-3 as an alternative marker for neutrophils and demonstrated a significantly increased fraction of positive cells in the livers of obese patients with NAFLD. Does Defensin 1-3 expression quantify neutrophil numbers or activation? Also, nitrosamine staining was not quantified as originally requested.

The Defensin quantify neutrophils number. We now included this information in the text. We used this marker because other reviewer mentioned that nitrotyrosine could be unspecific. We also included the quantification of nitrotyrosine Appendix S6.

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OK we added now this possibility in the text.

9. Figure 6F indicates that p38γ/δ-deficient neutrophils displayed increased rolling and decreased adhesion, which correlated with higher L-selectin and lower CD11b expression. This section would be substantially improved if the functional importance of p38γ/δ-dependent alterations in L-selectin and CD11b expression levels for neutrophil rolling/adhesion were tested directly (by knockdown / over-expression).

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We have now studied whether p38δ is deleted or not in macrophages. As expected, in the neutrophil-specific conditional mice p38δ is not deleted in bone marrow derived macrophages. Appendix S9.

10. Anti-Ly6G treatment significantly reduced triglycerides and ALT concentrations in both control Lysz-Cre and p38γ/δLyssz-KO mice (Figure 7 C and D). This would appear to indicate that neutrophils were required for MCD-induced liver steatosis and necrosis whether or not p38γ/δ was expressed in myeloid cells.

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3rd Editorial Decision   22 December 2015

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Thank you for your contribution to The EMBO Journal and congratulations on a successful publication.